FEBS Letters 426 (1998) 352–356 FEBS 20117

Composition of parental mitochondrial DNA in cloned bovine embryos

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Received 16 March 1998

Abstract We have investigated parental mitochondrial DNA (mtDNA) in cloned bovine embryos obtained by intraspecific cytoplast-blastomere fusion. Analysis of two-cell to blastocyst stage embryos revealed that in contrast to the exclusion of paternal (sperm) mtDNA during sexual inheritance in the cytoplast-blastomere fusion complexes, there was mixing and co-existence of parental mtDNA. The mixing of mtDNA was non-balanced with the minority deriving from the blastomere. The constant content of mtDNA during embryogenesis until the blastocyst stage suggesting an absence of mtDNA replication was shown for conventional 'in vitro fertilised' (IVF) embryos and for cloned embryos. The ratio of parental mtDNA was in accordance with the estimated quantitative participation of mtDNA from the fusion partners.

Key words: Cloning; Bovine; Mitochondrial DNA; Fusion; Allele-specific quantitation; TaqMan™ polymerase chain reaction

1. Introduction

Cloning by cytoplast-blastomere fusion is based on the transfer of whole blastomeres (nucleus donor) from cleavage stage embryos to enucleated meiotic metaphase II oocytes (cytoplast). The cytoplast which includes cytoplasmic organelles like the smooth and the rough endoplasmic reticulum, Golgi, tubules and other maternal factors being important for cell–cell communication and oocyte development is involved in the reprogramming of the introduced nucleus. The cytoplast's mitochondrial DNA (mtDNA) type is assumed to be transmitted to the cloned offspring [1]. However, in the cloning process there are two sources, i.e. cytoplast and blastomere, of parental mtDNA in the context of one nuclear genome which is surrounded by two types of cytoplasm. The transmission of the donor blastomere(DB)-mtDNA is not known.

The objective of this study was to monitor the fate of both types of parental mtDNA in cloned embryos of early developmental stages which were produced by intraspecific cytoplast-blastomere fusion. We did not consider from which breed the cytoplasts used in the fusion experiments were derived since fixed breed-specific mtDNA polymorphisms are not described for the population of European cows [2]. Another issue was to analyse the mtDNA content during early embryogenesis in cattle using in vitro fertilised and cloned embryos. The composition and transmission of parental mtDNA were investigated on the basis of polymorphisms in the control region (CR) of the mitochondrial genome.

2. Materials and methods

2.1. Oocyte maturation, donor embryos, recipient cytoplasts and embryo transfer

In vitro production of morula stage embryos, cytoplast-blastomere fusion and in vitro culture were carried out as described previously [3,4]. Bovine ovaries collected at the slaughterhouse originated nearly exclusively from Simmental cows (>95%), the remaining percentage from Holstein-Friesian and Brown Swiss. Female donors were not superovulated. We did not detect any phenotypic variation in donor oocytes between different breeds. In a series of single fusion experiments recipient cytoplasts (RCs) were derived from different ovaries. This approach resulted in an adequate number of oocytes with multilayered, compact cumulus and evenly granulated dark cytoplasm. Moreover, for molecular genetic analysis the use of RCs from different ovaries increased the chance to detect polymorphisms in the parental mtDNA. In our cytoplast-blastomere fusion experiments day 6 morulae were used. About 80% of the blastomeres derived from day 5 and day 6 bovine embryos have been shown to be in the S-phase [5]. Enucleated metaphase II oocytes used for fusion were preactivated by exposure to room temperature for 3 h prior to fusion (ageing). This step is expected to lead to recipient cytoplasts being also in the S-phase [6], i.e. to a synchronisation of blastomeres and cytoplasts. No blastocoele cavity was found in any of the embryos used in this study.

2.2. mtDNA analysis of cloned embryos of early developmental stages

In two sets of fusion experiments (set I and set II) individual donor blastomeres (DBs) were separated from a 58-cell morula and a 36-cell morula, respectively. The donor embryos used in set I and set II originated from Simmental cows. Set I resulted in the cloning of 11 embryos, three of which showed further development and were analysed at the 2-cell, morula and blastocyst stage. Set II yielded 15 cloned embryos, four of them were analysed at the 5-cell, 10-cell, 12-cell and blastocyst stage. Total cellular DNA was isolated from blastomeres and cloned embryos and was finally dissolved in 50 μl buffer as described [7]. Two single cells of the donor embryo which were isolated separately served as independent DB-mtDNA samples to ensure the validity of our experimental conclusions. Issues of cell size and DNA content in a single blastomere are very important. Two representative blastomeres which were comparable with the normal size of blastomeres at a certain morula stage were visually selected. The RC-mtDNA was amplified from the removed nucleus which also contained parts of the cytoplasm. Assuming an equal distribution of mitochondria in the ooplasm, the amount of mtDNA in RCs was evaluated by visually assessing the size of the remaining cytoplasm after enucleation of the oocyte (90–95%). This approach did not con-

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Abbreviations: DB, donor blastomere; RC, recipient cytoplast; CR, control region; AS-PCR, allele-specific PCR; NTC, no template control; NAC, no amplification control; T_a, annealing temperature; PIRA, primer introduced restriction analysis

PII S0014-5793(98)00350-0

sider the fact that mitochondria might be distributed unequally in the cytoplasm of bovine oocytes and may be associated with either the nucleous membrane or the cell membrane according to the cell cycle or other factors.

2.3. IVF embryos

In order to investigate the mtDNA content during early embryogenesis the following in vitro fertilised (IVF) embryos were analysed at the metaphase I and II stages, 2-cell, 8-cell, morula and blastocyst stage. Oocytes were derived from slaughterhouse material of one cow. In vitro culture and in vitro fertilisation were carried out as described previously [3]. Total cellular DNA was isolated as described [7] and was dissolved to give a final buffer volume of 50 μl.

2.4. mtDNA polymorphisms

Parental mtDNA was differentiated by screening the control region (CR) for single nucleotide substitutions. This region is known to undergo mutation readily [8,9]. From each sample of parental mtDNA we sequenced a 974-bp mtDNA fragment amplified with oligonucleotides CO1 and CO3 (see below) and subcloned into the pTAg vector (R & D Systems, Germany). For each parental type of mtDNA we sequenced two independent polymerase chain reaction (PCR) products by automated sequencing (LI-COR, USA) to confirm the point mutations used for allele-specific PCR (AS-PCR) or primer introduced restriction analysis (PIRA).

2.5. PCR and oligonucleotides

PCR amplification was carried out in a buffer containing 60 mM Tris-HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$, 0.2 mM dNTPs, 1 μM of each primer, 2 units *Taq* polymerase (Life Technologies, Austria) and 1 μl template DNA. PCR amplification was performed in a 50-μl reaction volume on a RoboCycler® (Stratagene, USA). The amplification was carried out under hot start conditions applying Hot-wax beads® (Invitrogen, The Netherlands) which contained $MgCl_2$ giving rise to final concentrations of 1.5, 2.5 or 3.5 mM or without 'hot start' using mineral oil instead of Hot-wax beads®. The individual annealing temperatures and $MgCl_2$ concentrations are indicated for each experiment. Each experiment included a no template control (NTC). The amplification was carried out routinely according to the following scheme: first cycle: 5 min 95°C/1 min at annealing temperature (T_a)/3 min at 72°C; 38 cycles: 20 s 95°C, 40 s at T_a/60 s 72°C; and last cycle: 20 s 95°C, 40 s at T_a/180 s 72°C. If not otherwise stated the products were analysed on ethidium bromide-stained 2% agarose gels. In the case of minor signals fragments were stained with SYBR Green I (FMC Bioproducts, Denmark). The size of the PCR products was determined on the basis of the localisation of primers on the reference sequence and by use of a molecular weight standard (100 bp DNA Ladder, MBI Fermentas GmbH, Germany).

PCR was performed with the oligonucleotides (CO1 to CO3) and allele-specific primers (AS1, AS3, and AS4) summarized in Table 1 which includes details about primers and probes used in the PIRA and TaqMan™ assays (see below). Their localisation in the bovine mtDNA CR is given in Fig. 1. As a control for the intactness of template DNA the whole CR of bovine mtDNA was amplified in parallel (primers CO1 and CO3, T_a= 48°C, 1.5 mM $MgCl_2$, hot-start PCR) to the allele-specific PCR of interest in a second set of tubes. Simultaneous amplification was not performed due to the limited amount of biological material to establish a multiplex assay.

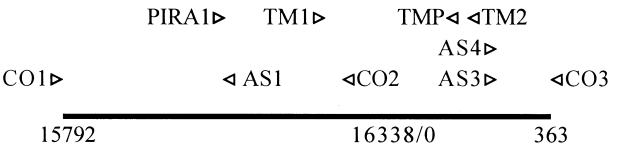

Fig. 1. Localisation of oligonucleotides (not true to scale, details in Table 1). The bold line indicates the CR of bovine mtDNA located between nucleotides 15792 and 363 (910 bp).

Allele-specific primers were designed instead of conventional oligonucleotides to exclude a possible amplification of a false 'mitochondrial allele'. Therefore, an additional mismatch at position 3 from the 3′ end of each primer was introduced [10,11]. Details concerning the design of allele-specific primers are given in Fig. 1.

2.6. Analysis of the distribution of parental mtDNA in early cloned embryos by PIRA

The A to G transition at nt16022 allowed the differentiation of parental mtDNA used in set I (Table 2). The rare allele 16022A present in the DB-mtDNA created a potential restriction site for *Nsp*I which was used for primer introduced restriction analysis. The primer PIRA1, possessing a mismatch at position 3 from the 3′ end, generated a *Nsp*I site in the PCR product amplified with PIRA1 and CO2 (T_a = 41°C, 1.5 mM $MgCl_2$). In the last two cycles 1 μCi of [α-^{32}P]-dCTP was added to label the PCR product. 15 μl of this reaction was cut with 2 U *Nsp*I (Life Technologies, Austria) for 3 h and run on a 10% non-denaturing polyacrylamide gel (Fig. 4B). The DB-mtDNA was taken as a control for a complete restriction digest. Gels were analysed on a Bio-Rad MG525 phosphorimager (Bio-Rad Laboratories, Austria).

2.7. Real-time quantitative PCR (TaqMan™ PCR)

The TaqMan™ PCR (PE Applied Biosystems, Germany; [12–14]), i.e. real-time quantitative PCR, was used to quantify the amount of mtDNA. It was carried out in 50 μl buffer volume containing 60 mM Tris-HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$, 0.2 mM dNTPs, 300 nM of each primer, 200 nM of the fluorogenic probe, 2 units of *Taq* polymerase (Life Technologies, Austria), 4 mM $MgCl_2$ and 1 μl template DNA. The ABI PRISM™ Sequence Detection System 7700 (PE Applied Biosystems, Germany) was used to detect the fluorescent signal which reflects the exponential accumulation of the PCR product. The threshold cycle values were determined using the Sequence Detector 1.6 software (PE Applied Biosystems, Germany). The fluorogenic probe (TMP) specifically anneals between the forward (TM1) and reverse (TM2) primer sites (Fig. 1). TaqMan™ PCR was performed according to a two-step PCR protocol (5 min at 95°C, 40 cycles for 15 s at 95°C and 1 min at 59°C) using the primers TM1 and TM2, and the probe TMP. A hot-start PCR was not necessary under these conditions. The probe and both TaqMan™ primers were of high-performance liquid chromatography (HPLC)-grade. To test the reliability of TaqMan™ PCR in each experiment the amplification of each sam-

Table 1
Oligonucleotides for PCR analysis

Oligo	5′-3′ sequence	5′-3′ position
CO1	cac cat caa ccc cca aag ct	15747–15766
CO2	cct gaa gaa aga acc aga tg	16284–16265
CO3	ttg ggt taa gct aca tca ac	383–364
AS1	gta ctt gct tat atg cat ggT(c) gt	16044–16022
AS3	cca gca taa tga taa C(g)ca	152–169
AS4	gag cac cag cat aat gat aaA(g) cg	147–169
PIRA1	atg tat ata gta cat taa att aC(t)a t	15997–16021
TM1	ctt aat tac cat gcc gcg tga	16159–16179
TM2	cca gct aca ata gat gct ccg	131–111
TMP	ttg acg gcc ata gct gag tcc	99–79

The position refers to the numbering in the GenBank Acc. No. V00654. Nucleotides generating a mismatch in the template DNA are printed as capital letters. The corresponding nucleotide of the reference sequence is given in brackets. The nonextendible hybridization probe TMP consists of an oligonucleotide with the 3′-quencher dye TAMRA (6-carboxytetramethylrhodamine) and the reporter dye FAM (6-carboxyfluorescein) attached to the 5′ end.

ple was repeated three times using the same master-mix. The threshold cycle (C_T) which is defined as the cycle at which each PCR amplification reaches a significant (i.e. usually 10 times the standard deviation of the baseline) threshold, is therefore given as a mean value. The real-time PCR method allows for a very large assay dynamic range (approaching 10^6-fold starting target). C_T values correlate linearly with relative DNA copy numbers [14].

3. Results and discussion

3.1. Cloned embryos of early developmental stages

Two sets of cloning experiments, set I and set II, were used to investigate the mtDNA composition in cloned embryos of differing early developmental stages. RC- and DB-mtDNA were distinguished by sequencing (Table 2), AS-PCR and RFLP analysis (Figs. 2 and 4B). The mitochondrial genomes of the fusion partners were shown to be different (Table 2). As expected, RC-mtDNA was present in all embryonic stages examined (5-cell to blastocyst stage; Fig. 2a). The DB-mtDNA was also detectable in all analysed samples (1-cell to blastocyst stage; Fig. 2b,c). The mixing of parental mtDNA immediately after fusion was monitored in two 1-cell embryos of set II using a polymorphism at nt 169 (data not shown). The mixing of parental mitochondria was non-balanced with the majority deriving from the enucleated oocyte (see below and Fig. 4B). The predominant contribution of RC-mtDNA could be expected if mtDNA replication would be absent from early embryogenesis, since the enucleated oocyte contains most of the mtDNA in its cytoplasm while the blastomere contains only a fraction of the entire cytoplasm of an oocyte.

In the following the mtDNA content during early embryogenesis between the 1-cell and the blastocyst stage was determined by TaqMan™ PCR. A constant level of mtDNA suggesting an absence of mtDNA replication was found for bovine IVF embryos (Fig. 3). This is in accordance with data obtained from mice [15]. However, this obvious absence of mtDNA replication found in mouse and bovine embryos could also be explained by the simultanous replication and degradation of mtDNA occuring at the same rate and, therefore, the total quantity of mtDNA stays the same. The amount of mtDNA in the cloned embryos was also constant until the blastocyst stage (Fig. 4A).

Next the ratio of parental mtDNA in cloned embryos was determined by PIRA. It could be argued that due to a missing 'reprogramming' of DB mitochondria in the cloned embryos one would expect their mtDNA to replicate earlier. However, we found a constant ratio of parental mtDNA in the cloned embryos of set I (Fig. 4B). The percentage of the DB-mtDNA is only 13%, 13% and 9% of the whole cloned embryo for the 1-cell stage, the morula and the blastocyst, respectively. The RC-mtDNA is the major type of mtDNA in the clones. Therefore slight changes in the amount of RC-mtDNA caused by the enucleation procedure (Section 2) are expected to have only a slight influence on its amount in the clones. This could explain the slightly lower value in the blastocyst.

In the case of neutral segregation the ratio of parental mtDNA can be explained by a simple model. This model takes into consideration the absence of replication during early embryogenesis (Fig. 4) and proposes a nearly similar partitioning of mitochondria into the dividing cells. It yields a theoretical percentage of 6.2%, 3.1% and 1.5% DB-mtDNA for clones derived from a 16-cell, 32-cell, and 64-cell donor embryo, respectively. In brief, the percentage of DB-mtDNA decreases with increasing number of blastomeres in the morula stage donor embryo, since the amount of mtDNA during

Table 2
Nucleotide positions divergent from the bovine mtDNA reference sequence

	sample	16022	16058	16156	16200	16302	16316	163	169	173	216+1	234	352+1	352+2	363
set I	DB-58-cell	A							G		C				
	2-cell (RC2)						C	G							
	morula (RC3)					A				G					
	blastocyst (RC4)								G						G
set II	DB-36-cell														
	5-cell (RC7)		T	T	A								C		G
	10-cell (RC8)										C	C	C		G
	12-cell (RC9)										C	C		C	G
	blastocyst (RC10)										C	C			G

The corresponding GenBank accession numbers for samples arranged from top to bottom are: AF022916, U92240, AF022917, AF022918, V00654, U92241, and AF022919 to AF022921. +1 and +2, insertion of one or two nucleotides.

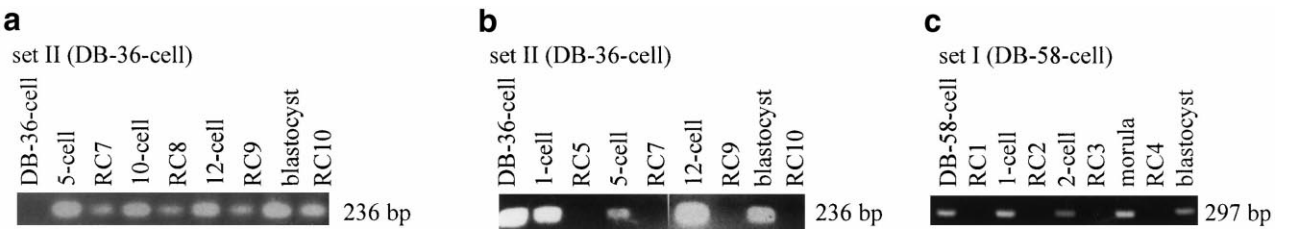

Fig. 2. Composition of parental mtDNA in cloned embryos of early developmental stages analysed by AS-PCR. Two sets of fusion experiments – set II (a and b) and set I (c) – were analysed. The donor embryo in each set originated from Simmental cows and the RCs from slaughterhouse material. Parental mtDNA was differentiated by sequencing. The RCs and the developmental stage of the cloned embryos is indicated above each lane. The number of cells of the morula stage of the two DBs is also given (DB-36-cell, DB-58-cell). Each experiment contained one NTC (not shown). a: Detection of RC-mtDNA in the cloned embryos of early developmental stages (primers CO3 and AS3, $T_a = 44°C$, 3.5 mM $MgCl_2$). b and c: Detection of DB-mtDNA in cloned embryos. A DB-specific fragment was found in the cloned embryos, but not in the corresponding RC-samples. b: Primers CO3 and AS4, $T_a = 54°C$, 1.5 mM $MgCl_2$. c: Primers AS1 and CO1, $T_a = 54°C$, 1.5 mM $MgCl_2$. The sample RC8, i.e. the RC used for cloning of the 10-cell embryo of set II, exhibited heteroplasmy at nt169 (data not shown) and was therefore not included in c.

early embryogenesis remains constant (Figs. 3 and 4). We conclude that all mitochondria and mtDNA alleles contained in these early cloned embryos show neutral segregation in relation to the nuclear background of the donor blastomere and to the initial surrounding of the RC which was expressed by the oocyte.

3.2. Embryo manipulation and mtDNA heteroplasmy

Our intraspecific fusion experiments revealed mixing and co-existence of parental mtDNA molecules in intraspecific crosses (Figs. 2 and 4B), i.e. a heteroplasmic population of mtDNA molecules was generated.

It is unknown whether the mtDNA heteroplasmy present in the cloned embryos which we have analysed, exists within a single mitochondrion, or reflects interorganellar heteroplasmy. The intraorganellar type of heteroplasmy has been suggested as cause for persistent heteroplasmy in slowly segregating human lineages, in cell culture models using transformants obtained from fusions between mtDNA-less (ρ^0) cells and human heteroplasmic cells and in a mouse lineage produced by embryonic karyoplast transplantation ([16], and references therein).

Data concerning the transmission of parental mtDNA after manipulation of mammalian embryos is still controversial. Karyoplast and cytoplast embryo reconstructions between zygotes carrying mitochondria of *Mus musculus molossinus* and *domesticus* subspecies have previously been shown to enable the production of animals with low levels of mtDNA heteroplasmy [17]. Heteroplasmic mice can also be created by intraspecific fusion of an enucleated zygote to a 1-cell embryo [18,19] or by karyoplast transplantation [16], but when mitochondria from liver or testis are microinjected into mouse zygotes, the exogenous mtDNA was not detected

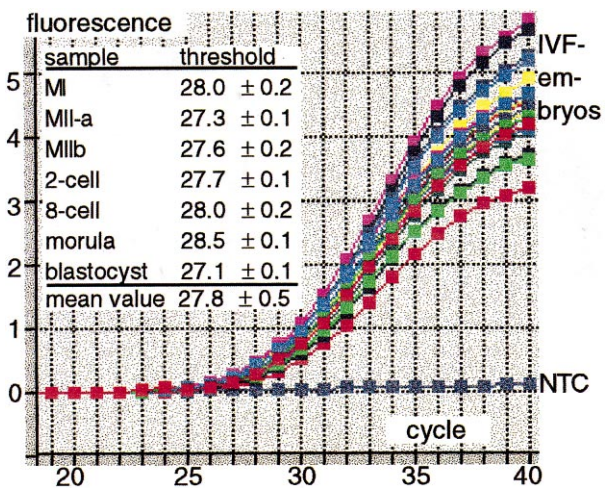

Fig. 3. Constant content of mtDNA in in vitro fertilised (IVF) embryos during early embryogenesis. The total amount of mtDNA in several embryonic stages was quantified by TaqMan™ PCR. The threshold cycles for each embryonic stage were determined in triplicates. No signal was detected in the NTC sample (no template control). The first PCR cycles are not shown since the fluorescence signal was still below the threshold for detection. Individual amplification curves are not specified due to the small overall standard deviation (=0.5) of threshold cycle values, and due to the fact that the endpoint values of amplification curves are not considered in real-time detection. MI and MII, metaphase I and II stages.

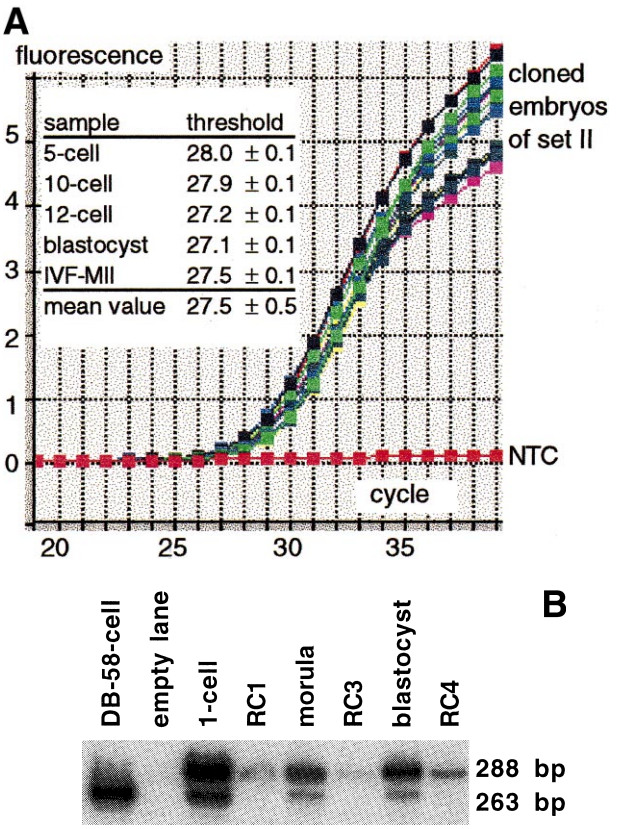

Fig. 4. Parental mtDNA of set II (A) and set I (B) early cloned embryos is neither replicated nor excluded (for details see Section 3). The RCs and the cellular stages of the DBs are given above each figure. A: Absence of replication is shown by TaqMan™ PCR. The four embryonic stages investigated contained the same total amount of mtDNA as a metaphase II stage IVF embryo. The first PCR cycles are not shown since the fluorescence signal was still below the threshold for detection. Individual amplification curves are not specified due to the small overall standard deviation (=0.5) of threshold cycle values, and due to the fact that the endpoint values of amplification curves are not considered in real-time detection. B: Maintenance of RC- and DB-mtDNA. Parental mtDNA which exhibited an A to G substitution at nt 16022 was analysed by PIRA (primers CO2 and PIRA1, T_a = 41°C, 1.5 mM $MgCl_2$, *Nsp*I restriction digest). PCR products were radioactively labeled prior to digestion as described in Section 2 and separated on a 10% polyacrylamide gel. The amount of the digested and undigested products was quantified by phosphorimager analysis. For abbreviations see Fig. 2. Signal intensity in all RC lanes is lower than that of the different embryonic stages since the amount of mtDNA used for PCR was only about 5% to 10% (see Section 2). The percentage of the DB-mtDNA compared to the RC-mtDNA was 13%, 13% and 9% for the 1-cell stage, the morula and the blastocyst, respectively. The mtDNA in the 2-cell embryo of set I degraded soon after the first set of PCR amplifications (Fig. 2) and failed in the PIRA assay presented here.

in the progeny [20]. Recently, in mice foreign mitochondria isolated from somatic cells were introduced by interspecific microinjection into recipient germline cells and were detectable at least still at the blastocyst stage ([21], see also Lathe and Pinkert et al., Letters to the Editor, Transgenic Res. 7, pp. 3–4). For further discussion we refer to Steinborn et al. (this issue).

Whether the heteroplasmic population of parental mtDNA generated by cytoplast-blastomere fusion in this study can also be found in cloned cattle after birth merits investigation.

Acknowledgements: We thank B. Salmons and W.H. Günzburg for valuable discussion and comments on the manuscript. We also acknowledge R. Badegruber and M. Nowshari for the production of IVF embryos. We are grateful to T. Schild (PE Applied Biosystems) and W.H. Günzburg for their support of TaqMan™ PCR. Financial support was provided by the Austrian Ministry of Agriculture.

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